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TEMPERATURE DEPENDENT CILIARY RHYTHMICITY IN *MYTILUS EDULIS* AND THE EFFECTS OF MONOAMINERGIC AGENTS ON ITS MANIFESTATION

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Some ciliated cells are directly influenced by temperature changes. In *Mytilus edulis* the same relationship can be demonstrated for frontal cilia of isolated gill filaments (Gray, 1929; Hirasaka, Hoshi and Nagumo, 1957; Hoshi and Hoshiyama, 1963). Lateral cilia of isolated gill of *M. edulis* exhibit this temperature effect only if they are first stimulated to beat by the addition of exogenous agents (Aiello, 1960). The need for artificial stimulation to induce lateral ciliary activity of isolated, denervated gill suggests the presence of endogenous innervation mechanisms.

Lateral ciliary activity of *M. edulis* is known to be peripherally dependent on serotonin and dopamine (Aiello, 1962, 1970; Gosselin, 1961; Gosselin, Moore and Milton, 1962; Aiello and Guideri, 1964, 1965, 1966; Paparo and Aiello, 1970). Electrical stimulations of the branchial nerve can either excite or depress lateral ciliary activity (Aiello and Guideri, 1966; Paparo and Aiello, 1970). Recent morphological pharmacology studies (Stefano and Aiello, 1975; Stefano, Catapane and Aiello, 1976) and investigations into the central nervous system (CNS) regulation of ciliary activity (Catapane, Aiello and Stefano, 1974; Catapane, 1976; Catapane, Stefano and Aiello, 1976) have shown that the CNS is composed in part of serotonergic and dopaminergic neurons. The majority of the serotonin content of gill is dependent upon axonal transport of tryptophan hydroxylase from the CNS *via* the branchial nerve. Environmental temperature changes produce changes in endogenous serotonin levels which appear to change first in the CNS (Stefano and Catapane, 1977a). The evidence suggests a dual antagonistic innervation of the lateral ciliated cells originating from the cerebral and visceral ganglia.

The present study sought to investigate the nervous control of lateral ciliary activity in nature as well as in the laboratory. The possibility of rhythmic activity was examined, along with several environmental factors which may influence it.

Materials and Methods

Field study

Subtidal specimens of *M. edulis* were observed *in locus* at Island Beach State Park, New Jersey, by means of scuba diving. During dark hours a portable underwater light was used. Slightly open animals were selected for use. Their

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posterior adductor muscle was severed while underwater, and the animals were dislodged from their clusters. Jørgensen (1975) has shown that cutting the posterior adductor muscle does not impair the health of the animals nor effect the rate of water transport by the gill. At the time of collection, the water temperature at the level from which the animals were harvested was recorded and a quantity of this water was taken in which to transport them. The delay between the time of collection to the time of observing ciliary activity was approximately 30 min, during which time the animals remained at a constant temperature in five gallons of sea water gathered from their level. Animals were collected and ciliary rates observed hourly for 24 hour periods on June 10, 21 and August 23 and at irregular intervals during the months of June, July and August.

When pharmacological pretreatments were part of the experiments, animals were injected into the posterior adductor muscle while underwater. The drug solution was prepared and carried in labelled syringes prior to diving. Each drug was administered in a 0.1 ml volume of filtered sea water. Color coded nylon threads were used to identify animals and drug treatments. At the appropriate times, animals were transported quickly to the nearby observation site, where lateral ciliary activity was observed stroboscopically according to the method of Gray (1930) and Dral (1967).

Laboratory study

Specimens of *M. edulis* were collected at Long Island Sound, New York, at New Rochelle, transported to the laboratory and maintained in artificial sea water (ASW) prepared from Instant Ocean Sea Salts, at a pH of 7.0–7.4, specific gravity of 1.022–1.026, with a constant aeration supply and varying temperature and time periods depending upon the experiments.

Whole animal preparations consisted of deshelled animals positioned in petri dishes. The mantle and the lateral gill lamina from the right side of each animal were excised to allow the cilia on the medial lamina to be viewed with transmitted stroboscopic light. The cerebral and pedal ganglia remained connected to the visceral ganglia and the gill; however, the visceral commissure was transected. These whole animal preparations maintained all neuronal connections except those linking the two visceral ganglia. The beating rate of each preparation was measured each minute for ten minute periods. The mean $\pm$ s.e.m. of the values were calculated from all the preparations observed for each type of experiment. During the time course of the observation periods, the temperature of the medium bathing the animals did not vary by more than 2° C.

Visceral ganglion (VG) preparations were composed of only the gill and the visceral ganglion connected by the branchial nerve. Electrical stimulations were delivered by platinum needle electrodes with a Harvard 342 Stimulator. Repeating bipolar pulses at 0.1 Volts and 5 or 50 Hz for three minutes were applied to the branchial nerve.

Observations and Results

Field study

Specimens of *M. edulis* observed underwater had their valves slightly open for the majority of the time they were viewed. They would close their valves in

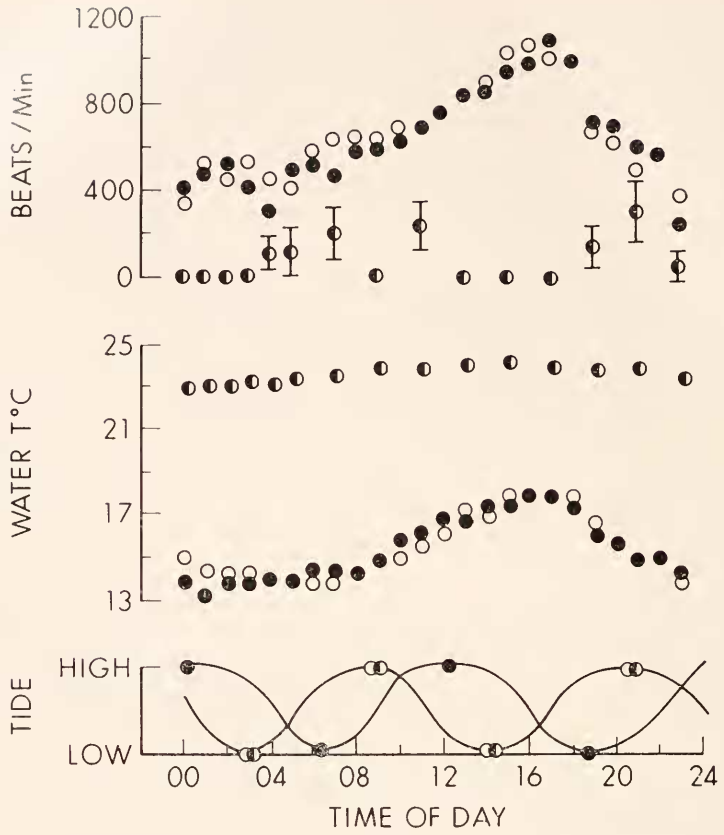

FIGURE 1. Graph showing the relationships between tides, water temperature (°C) and beating rates (beats/min) of lateral cilia of whole animal preparations of *M. edulis*. Open circles represent June 10, solid circles June 21, and half solid circles August 23. The tidal cycles for June 10 and August 23 are almost identical, so only one line is shown. The beating rates for each hour represents the mean of the average of 10 one minute interval readings/animal for five to ten animals. In all cases except where a bar shows s.e.m., the s.e.m. was less than 10% of the beating rate.

response to a disturbance and reopen them at a later time. Those animals which were collected hourly for 24 hour periods and had their lateral ciliary activity assessed showed a diurnal ciliary rhythm (Fig. 1). For June 10 and 21 the activity appeared to be independent of tidal cycles, but rose and fell in coordination with the daily rise and fall of the water temperature and the sun. For August 23 no ciliary rhythmicity was observed, and the water temperature remained fairly constant. To examine environmental influences more closely, groups of animals were collected between June 6 and July 27, on 16 different occasions at different tidal periods, and their beating rates were plotted against the tidal period and temperature at which they were collected (Fig. 2). Examination of these figures reveals that a linear regression with a correlation coefficient of 0.8 can be plotted

for the plot against temperature, but the plot against tidal period does not show a corresponding relationship.

In order to determine the influence of the nervous system on the rhythmicity of ciliary activity, animals were injected with neurotransmitters and drugs which affect neurotransmitter metabolism. Animals for similar experiments were uniformly treated, collected and observed at the same time so that the behavioral modifications were not due to different groups being collected at different periods of their temperature-activity cycles.

The lateral cilia of whole animal preparations were observed after quickly transporting them from their ocean environment to the field observation station. Prior to their collection, the posterior adductor muscle of slightly gapping animals was injected *in locus* with 200 μg of serotonin, dopamine or acetylcholine or 100 μg of 6-hydroxydopamine (6-OHDA), alpha methyl-para-tyrosine (AMPT) or 5,6-dihydroxytryptamine (5,6,-DHT) dissolved in 0.1 ml of filtered sea water for varying time periods. Controls were vehicle injected. No mortalities were observed for at least two weeks as a result of these injections.

Table I shows that the average basal activity of the control group was 562 beats/min. Those animals which were pretreated two hours earlier with serotonin displayed substantially higher basal beating rates. The mean rate was 1039 beats/min, about twice the value of untreated animals. Animals injected with dopamine for the same two hour interval were not beating at all. The animals injected with acetylcholine did not differ in their activity from that of the controls.

Another set of animals were observed four and six hours after injections. They displayed similar activity as did the two hour groups. Table I shows the basal beating rates of sets of animals observed 17 and 21 hours after injections. The control groups were beating at 740 for the 17 hour reading and 848 beats/min for

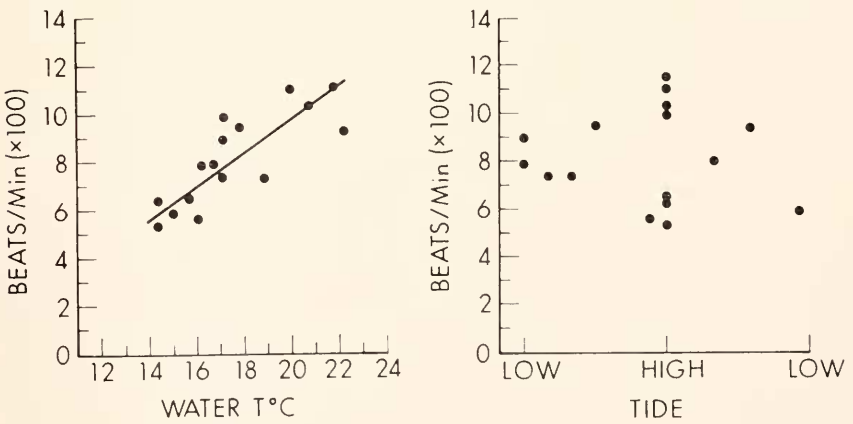

FIGURE 2. Lefthand graph shows the relationship between the temperature at which the animals were collected and lateral ciliary beating rates (beats/min) of whole animal preparations. Each point represents the mean values of five animals. The s.e.m. for each point is less than 10% of the beating rates. The best fit line is drawn on the graph. The correlation coefficient r is 0.82. In the righthand graph, the same beating rates are plotted against the cycle of the tide at which the animals were collected. No correlation exists.

Table I

Lateral ciliary activity (beats/min ± s.e.m.) of whole animal preparations. Groups of animals were injected while underwater with the indicated drugs and their cilia observed at the specified time intervals; 200 μg of serotonin (HT), dopamine (DA) or acetylcholine (ACH) were injected into the posterior adductor muscle in a 0.1 ml volume. Control animals were injected with 0.1 ml of sea water. N is the number of animals for each treatment.

Treatment	Time after injection (hr)	N	Ciliary rate (beats/min ± s.e.m.)
SW	2	5	562 ± 43
HT	2	5	1039 ± 24
DA	2	5	0
ACH	2	5	608 ± 16
SW	6	5	943 ± 33
HT	6	5	1173 ± 25
DA	6	5	0
ACH	6	5	932 ± 33
SW	17	5	740 ± 23
HT	17	5	1024 ± 56
DA	17	5	0
ACH	17	5	821 ± 40
SW	21	5	848 ± 16
HT	21	5	1194 ± 73
DA	21	5	620 ± 73
ACH	21	5	790 ± 29

the 21 hour reading. Those animals which were injected earlier with serotonin maintained high basal rates. At 17 hours it was 1024, and at 21 hours it was 1194 beats/min. The dopamine-injected animals had cilia which were still quiescent after 17 hours. For the group injected group injected 21 hours earlier with dopamine, the cilia were beating at 620 beats/min. Animals injected with acetylcholine did not display any altered ciliary activity at any of the time intervals at which they were observed.

Animals were administered 6-OHDA and AMPT which specifically interfere with dopaminergic mechanisms, and 5,6-DHT which interferes with serotonergic systems in an attempt to interfere with monoaminergic mechanisms. Because these agents exert neurotoxic and metabolic effects, longer time periods were necessary to observe the behavioral effects. The whole animal preparations showed altered ciliary activity in response to each drug treatment (Table II). Basal activity of animals observed four days after injection with 6-OHDA was 1002 beats/min as compared to 603 for the vehicle injected controls injected at the same time. The basal rate of animals injected with AMPT was 904 beats/min as compared to controls which were beating at 475. The set of animals which were injected with 5,6-DHT three days earlier were not beating when observed. The controls for this experiment were beating at 576 beats/min.

Electrical stimulation to the branchial nerve can elicit cilio-excitation or inhibition depending on the stimulus parameters employed (Aiello and Guideri, 1966; Paparo and Aiello, 1970). The cilio-excitatory response is dependent on peripheral release of serotonin, while the inhibitory response is due to dopamine. Therefore, the effects of branchial nerve stimulation in animals pretreated with

6-OHDA, AMPT or 5,6-DHT were studied. VG preparations were chosen for these studies so that incoming nervous activity from the cerebral ganglia would not interfere with and obscure the interpretation of the results and so that the preparations conformed to the type used by the earlier works which established the stimulus parameter relationships. For each preparation the branchial nerve was stimulated with a 5 or 50 Hz bipolar 2 msec duration pulse and 0.1 Volts for 3 min, after observing basal rates.

While whole animal preparations tend to display spontaneous activity, a higher number of VG preparations tend to be inactive. Figure 3 shows that for control animals, stimulations at 5 Hz substantially accelerated beating, while stimulations at 50 Hz decreased ciliary rates. The response to 50 Hz stimulation is not always seen for all the gill filaments in the field of view. However, those filaments that do respond, only decrease their beating rates after a few second latency period, and repeatedly do so in response to repeated stimulations at later times.

Animals which were pre-injected four days earlier with 6-OHDA displayed an altered response to stimulations (Fig. 3). When 5 Hz was employed, they increased their beating rates from 0 to 800 beats/min within 3 min. When 50 Hz was employed, they only decreased their rates slightly to 630 beats/min as compared to 125 beats/min for the controls. The cilia of the control animals did not return to their basal rates during the observation period, while the cilia of the 6-OHDA treated animals did.

Animals which were treated four days earlier with AMPT were similarly tested. The cilia of the AMPT-treated animals responded almost identically as did the cilia of the 6-OHDA-treated animals (Fig. 3).

Animals which were injected three days earlier with 5,6-DHT had no basal ciliary activity. Stimulation at 5 Hz failed to activate the lateral cilia. However, the addition of 10^{-4} M serotonin to the gill medium activated cilia and increased beating rates to 1000 beats/min. Groups of animals were injected with 5,6-DHT and observed at varying time intervals between 12 hours and three days to find a period when the cilia were spontaneously active, to determine if 50 Hz stimulations would be inhibitory. Only the animals which were observed 12 hours after injections had beating rates above 0. For these animals, stimulations at 5 Hz

TABLE II

Lateral ciliary activity (beats/min ± s.e.m.) of whole animal preparations which were pretreated in locus *with 6-OHDA, 5,6-DHT or AMPT (100 μg in a 0.1 ml volume) for the indicated time periods prior to observation. Control animals were injected with sea water. N is the number of animals per group. Statistical significance was determined by a one tailed Student's* t-*test.*

Treatment	Time after injection	N	Ciliary rate (beats/min ± s.e.m.)
6-OHDA	4 days	5	1002 ± 44*
SW	4 days	5	603 ± 38
5,6-DHT	3 days	5	0*
SW	3 days	5	576 ± 56
AMPT	4 days	5	904 ± 53*
SW	4 days	5	475 ± 93

*$P < 0.001$

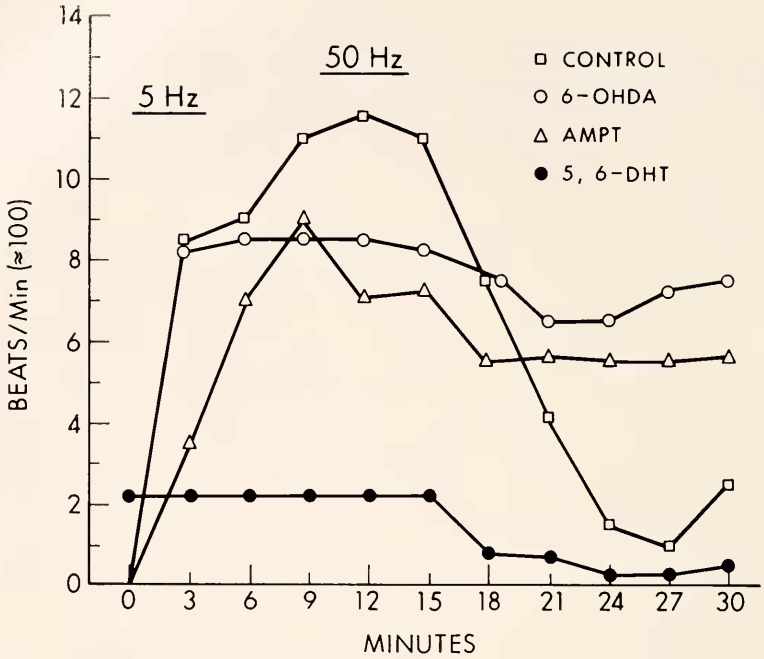

FIGURE 3. Lateral ciliary activity (beats/min ± s.e.m.) of VG preparations is shown. Each point represents the mean of five preparations. The preparations were observed for 15 min before starting the experiments. Groups of animals were preinjected 12 hr earlier with 5,6-DHT or four days earlier with 6-OHDA or AMPT. Electrical stimulation at 5 or 50 Hz was delivered to the branchial nerve at the indicated times. The s.e.m. of each point was less than 10% of the beating rates.

still failed to accelerate beating but stimulations at 50 Hz depressed the cilia (Fig. 3).

Laboratory study

Animals were collected from Long Island Sound during October and maintained at 5–6° C, 15–16° C and 20–24° C in ASW under otherwise identical conditions for six days. Table III shows that lateral ciliary rates are dependent on the temperature at which the animals were kept. All the preparations were observed at the same temperature, 20° C. Groups of animals that were kept at these temperatures but under constant darkness, except for irregularly spaced intervals when the water was changed, were also observed. Lateral ciliary beating did not vary in respect to different lighting cycles during the time course of the experiments.

DISCUSSION

The field experiments demonstrated that ciliary activity of whole animal preparations was not constant but exhibited a diurnal rhythm which was independent of the tides. The effects of temperature changes could not be distinguished from

those of light, since they both increased and decreased in unison during the study. The effects of different lighting cycles on this diurnal activity was examined in the laboratory, and it was found that depriving animals of light did not significantly alter ciliary activity. Therefore, the ciliary rhythm observed in nature corresponds with the changing environmental temperature and not with light.

The dependency of lateral ciliary activity on the peripheral transmitters, serotonin and dopamine, has been illustrated in numerous studies. Recent studies involved with the CNS regulation of ciliary beating has shown that both the cerebral and visceral ganglia exert influences on ciliary activity which can be elicited by the application of serotonin and dopamine to the ganglia (Catapane, Aiello and Stefano, 1974; Catapane, 1976; Catapane, Stefano and Aiello, 1976). Applications of serotonin and dopamine to the CNS increased or decreased ciliary rates, respectively. The animals which were injected with serotonin continually beat at higher rates than did controls for up to 21 hours. The animals injected with dopamine showed depressed activity throughout this time period, while the acetylcholine-injected groups showed no variance from controls.

Previously it has been shown by histofluorescence that injecting serotonin and dopamine into the posterior adductor muscle results in their selective accumulation into the nervous system (Stefano and Aiello, 1975). This increase was able to be visualized for at least two days after treatments. The prolonged presence of serotonin and dopamine in the animals had a physiological effect on ciliary activity. The origin of this neurological effect could be a combination of both ganglionic and peripheral actions.

Pharmacologically altering the animal's monoamine content resulted in modifications of the diurnal ciliary activity. Selectively depleting with AMPT (Underfriend, Zaltzonan-Nirenberg and Nagatsu, 1965) or destroying the dopaminergic system with 6-OHDA (Thoenen and Tranzer, 1968) caused an increase in the endogenous basal activity and, correspondingly, an impairment or complete disfunctioning of the inhibitory mechanism. Serotonergic destruction with 5,6-DHT (Baumgarten, Bjorklund, Lachenmayer, Nobin and Steneir, 1971) likewise changed the endogenous basal ciliary activity. Consistently, cilia of animals treated in this manner were quiescent or beating at low rates. Stimulating the branchial nerve did not produce cilio-excitation. However, addition of serotonin to the gill did increase beating rates, showing that the ciliated cell receptor itself was unaltered.

TABLE III

Lateral ciliary activity (beats/min ± s.e.m.) of whole animal preparations. Groups of animals were maintained for six days at the indicated temperature, either in a normal light-dark cycle or in the absence of light. N is the number of animals in each group. Statistically, there is no significance between the groups kept in the dark as compared to those with a light-dark cycle.

	Ciliary rates (beats/min ± s.e.m.)			
	N	Normal light-dark	N	Absence of light
5–6° C	5	0	5	0
15–16° C	5	978 ± 43	5	960 ± 78
20–24° C	5	1178 ± 12	5	1098 ± 35

Jørgensen (1975) concluded that serotonergic innervation plays no important role in regulating the frequency of lateral ciliary beating, but may only be concerned with maintaining lateral ciliary activity. He bases his conclusions on the fact that "the sensitivity (to serotonin) of both lateral cilia and laterofrontal cirri increased with age of the excised gill fragments until advanced stages of tissue disintegration" (Jørgensen, 1975, p. 224), and that serotonin did not change the rate of yeast clearance by undisturbed animals. The sensitivity phenomenon which he describes is exactly what would be expected as a result of supersensitivity from denervation produced by excising gill fragments. The relationship among lateral ciliary beating rates, rate of water pumping and rate of particle filtration has not been adequately described at this time, but is known to vary under differing conditions (Dral, 1967; Hildreth, 1976). Based upon these facts, the well known relationship between the rate of lateral ciliary beating and the dose of serotonin added to gill (Gosselin, 1961; Aiello and Guideri, 1966), and recent studies showing that the rate of lateral ciliary beating increases with increasing concentrations of serotonin applied directly to the visceral ganglion of preparations in which the media bathing the gill is maintained separately from that bathing the visceral ganglion (Catapane, Stefano and Aiello, in preparation), it appears that frequency of lateral ciliary beating may well be dependent upon the serotonergic innervation.

In *Anodonta cygnea,* the catecholamines and serotonin serve not only as peripheral neuroeffectors of the adductors but also in influencing the periodicity of the animals' activity (Salanki and Hiripi, 1970; Hiripi, 1973; Salanki, Hiripi and Nemcsok, 1974a,b). This activity was subject to changes by the use of various drugs (Hiripi, 1973).

Histofluorescent and biochemical evidence supports the proposed drug actions and physiological findings in our experiments. As shown previously, AMPT and 6-OHDA not only lowered dopamine content of the CNS but also produced a detectable rise in serotonin (Stefano, Catapane and Aiello, 1976). The sustained high beating rates and the inability to neuronally inhibit them must be viewed as a combination of a dopaminergic impairment and an increased serotonin content.

Several earlier studies examined the relationship between ciliary activity and temperature in *M. edulis.* These studies were concerned with frontal cilia and measured either particle movements (Gray, 1929, 1930), or gill crawling rates (Hirasaka *et al.,* 1957; Hoshi and Hoshiyama, 1963) of isolated gills. Aiello (1960) studied the temperature-activity relationship of lateral cilia in *M. edulis* and found a Q_{10} of 1.84 for beating rates *vs.* temperature over the range of 2–24° C. In his experiments, however, veratrine sulfate was always added to the gill bathing fluid to initiate and stabilize lateral ciliary activity. In muscular tissue, veratrine acts at the postjunctional and sarcoplasmic membranes to initiate excitatory postjunctional potentials (Goodman and Gilman, 1967, p. 604). Isolated (denervated) gills without this treatment or the addition of potassium tend to have no lateral ciliary activity or very poor and sporadic activity regardless of the temperature (Aiello, 1960; Takahashi and Murakami, 1968). In the present study, the CNS innervation of the gill was left intact. These preparations had stable-active ciliary rates which changed speeds in response to changing temperatures without the need of exogenous stimulations.

Selective destruction of the serotonin system produced by 5,6-DHT accounts for the inability of cilia to beat continuously and for the lack of response to neuronal stimulations. A previous study (Stefano, Catapane and Stefano, 1976) showed the involvement of serotonin in a temperature dependent ciliary rhythm. In the present study, pharmacological alterations of the serotonin and dopamine contents changed this temperature-dependent rhythm. Since isolated gill do not display ciliary rhythmicity with respect to temperature, unless the receptors of the ciliated cells are artificially activated, this indicates that central pathways are involved in the manifestation of this peripheral activity in the intact organism. The interrelationship of these pathways is in need of further investigation in terms of distinguishing central from peripheral mechanisms. Therefore, the natural lateral ciliary rhythm may be regulated in part by the nervous system responding to temperature changes and also may be the result of a direct effect of temperature on the sensitivity of the ciliated cell receptor to nervous stimulation.

In the field during the latter part of August and into September, a greater number of dead mussels were found than during other times of the summer. Cilia of healthy animals were either not beating or were slowly beating with no apparent rhythmicity. The water temperature was warm and in a 24 hour period it did not vary more than 2 degrees. These higher relatively constant environmental temperatures induced an overall higher metabolic rate which could not be nutritionally supported, or the high metabolic rate itself could not be sustained. Therefore, a possible explanation of this irregular activity pattern may be the overall poor health of the animals due to starvation. It has been a general observation in our laboratory that during February a large number of dead and unhealthy animals are seen at collection areas. At this time, the prolonged exposure to low temperature, which lowers CNS monoamine content (Stefano and Catapane, 1977b) and depresses ciliary activity, may also result in starvation.

In conclusion, *M. edulis* contains a temperature-sensitive neuronal mechanism involving a serotonergic system capable of altering ciliary rates. The possible effects of thermal pollution should be considered on the nervous system and overall health of coastal shellfish in view of the fact that constant high temperatures can cause starvation even if a plentiful food supply is available.

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Summary

Lateral ciliary activity of the bivalve mollusc *Mytilus edulis* was studied in the field and in the laboratory. A diurnal rhythm corresponding to the environmental

temperature changes was found. This behavior was modified by treating animals with serotonergic and dopaminergic agents, disrupting the serotonin and dopamine innervation of the cilia. The study shows that manifestation of this temperature-dependent rhythmicity is due peripherally and centrally to monoaminergic pathways.

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